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Importance of a Hydrophobic Pocket for Peptide Binding in Lactococcal OppA▽

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Lactococcal oligopeptide-binding protein A (OppA) binds peptides with widely varied lengths and sequences. We previously hypothesized that a hydrophobic pocket in OppA preferentially binds a hydrophobic peptide side chain and thus determines its binding register. Two crystal structures of OppA with different nonapeptides now indeed show binding in different registers.

Lactic acid bacteria (LAB) employ an active proteolytic system that allows them to utilize molten-globular milk proteins like α_{s1}, α_{s2}, κ, and β-casein as their sole source of nitrogen (amino acids) (15). A single cell wall-bound proteinase hydrolyzes these proteins to relatively long peptides, ranging in length from 6 to over 20 amino acids (9). Unlike enteric bacteria, LAB have the capacity to transport these long peptides into the cell, a property that is best documented for *Lactococcus lactis* (4, 5, 7, 9, 10, 15). The oligopeptide permease (Opp) plays a major role in oligopeptide uptake in *L. lactis* (9). Opp is an ABC transporter, composed of five subunits: two nucleotide-binding proteins (OppD, OppF), two transmembrane proteins (OppB, OppC), and a substrate-binding protein (OppA), which is anchored to the cell membrane via lipid modification of the N-terminal cysteine. OppA from *L. lactis* binds peptides of widely varied lengths, from 4 to at least 35 amino residues long, with little sequence selectivity. OppA delivers the bound peptides to the permease for transport into the cell. We recently determined the structural basis for peptide binding in OppA (2). The protein contains an exceptionally voluminous binding cavity (~4,900 Å^3) that can accommodate large peptides. Hydrogen bonds are formed between the protein and the peptide backbone, covering a stretch of 5 residues, which effectively determines the lower size limit of the substrates. Hydrogen bonds between the protein and peptide side chains are absent, explaining the lack of sequence specificity. The peptide termini, contrary to, for instance, those in crystal structures of OppA from *Salmonella enterica* serovar Typhimurium and DppA from *Escherichia coli* (14, 16), are not fixed in position with salt bridges, explaining how peptides of various lengths can bind to OppA from *L. lactis*. On the basis of the structures of OppA in its open-liganded and closed-liganded conformations, we proposed a new model for peptide binding (2). A key feature of the model is that different peptides of the same length may bind in different registers. The binding register is determined by a central, well-defined hydrophobic pocket in the binding site of OppA that prefers to accommodate a hydrophobic side chain of the peptide. To further demonstrate that peptides indeed bind in different registers, we crystallized OppA in the presence of two similar nonapeptides with a hydrophobic residue at position 5 or 6.

The sequences of the two nonapeptides are SLSQLSSQS and SLSQSLSQS, with the only difference between them being the position of the central leucine residue. The sequences are based on the casein-derived nonapeptide SLSQSKVLP, which is a natural substrate for OppA (10). The dissociation constants for binding to OppA are 1.2 μM and 4.2 μM for SLSQ LSSQS and SLSQSLSQS, respectively, determined by intrinsic protein fluorescence titrations as previously described (2; data not shown). To obtain peptide-bound protein crystals, OppA* (the asterisk indicates that the protein lacks the signal sequence and lipid modification) was expressed, purified, and crystallized, as previously described (2), with the protein drop supplemented with a 1 mM concentration of either peptide. Diffraction-quality crystals of peptide-bound OppA* were obtained after 6 months of incubation at 18°C. Data were collected at beamlines PXIII (SLS, Villigen, Switzerland) and ID14-1 (ESRF, Grenoble, France) (Table 1). Crystals of OppA* that cocrystallized with SLSQLSSQS and SLSQSLSQS diffracted to 2.9- and 1.5-Å resolutions, respectively. Data processing and reduction were carried out using XDS and programs from the CCP4 package (8, 3). The structure was solved by molecular replacement using Phaser (12), with the main chain of the previously determined structure of OppA* (Protein Data Bank code 3DRG) as a search model (no ligand was present in the search model). The output from Phaser was further refined using Refmac5 and Phenix.refine (1, 13), interspersed with manual building rounds in Coot (6). Both structures of OppA*, complexed with SLSQLSSQS or SLSQSL SQS, were in the closed conformation and very similar to OppA* with bound bradykinin (RPPGFSPFR) (root mean square deviation of 0.4 Å over values for all C-α atoms). During refinement, residual electron density appeared in the ligand-binding cavity in both structures at a position similar to

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TABLE 1. Data collection and refinement statistics[a]

Ligand	Value(s) for:	
	SLSQLSSQS	SLSQSLSQS
Data collection statistics		
Space group	$P2_12_12_1$	P1
Cell dimensions		
a, b, c (Å)	41.6, 99.6, 123.9	42.4, 58.9, 61.4
α, ß, γ (°)	90, 90, 90	100.6, 101.3, 103.4
Wavelength (Å)	1.0	0.872
Resolution range (Å)	46–2.9	37.6–1.5
No. of unique reflections	11,804	84,393
Completeness (%)	98.3 (97.1)	95.9 (93)
R_{meas}	0.23 (0.67)	0.05 (0.39)
I/σ (I)	7.8 (2.6)	14.3 (2.9)
Redundancy	4.0	2.0
Refinement statistics		
Resolution range	46–2.9	29–1.5
No. of reflections	11,802	82,534
R_{work}/R_{free}	0.22/0.26	0.16/0.19
No. of atoms		
Protein	4,429	4,510
Water	0	1,169
Avg no. of B-factors (Å^2)		
Protein	29.6	16.4
Water		28.4
RMS deviations		
Bond length (Å)	0.006	0.007
Bond angle (°)	0.948	1.120

[a] The numbers in parentheses correspond to the highest-resolution shell. RMS, root mean square.

that observed for bradykinin in the previously determined structure. After we finalized the refinement of the protein, the backbone and side chain electron density of the two residual densities were of sufficient quality to allow for modeling of SLSQLSSQS and SLSQSLSQS. After the final refinement, the positions of the peptides were verified by creating simulated annealing omit maps, using models where the ligands had been left out.

Superimposing the two OppA* structures revealed that the two peptides were bound by OppA* in different registers. The backbone of residues 2 to 9 of SLSQSLSQS superimposed on the backbone of residues 1 to 8 of SLSQLSSQS. In both structures, the central leucine residue of the peptides (positions 5 and 6 in SLSQLSSQS and SLSQSLSQS, respectively) occupied the well-defined, hydrophobic side chain pocket of OppA*. In the previously determined structure of bradykinin-bound OppA*, this pocket contained the side chain of phenylalanine at position 5 of the peptide. In the OppA*/SLSQS LSQS complex, the peptide's register thus shifted by 1 residue from the register of the OppA*/SLSQLSSQS and OppA*/bradykinin complexes (Fig. 1). This is consistent with our hypothesis that peptides of the same length and composition and with similar sequences can bind in different registers to OppA*. The change in register can be accommodated because the termini of the peptides are not fixed in position (see Fig. 2 for a schematic of the binding mechanism).

Our current knowledge that peptides of the same length can bind in different registers to OppA* stimulates a reinterpretation of previous results. Peptide binding to OppA* has previously been studied in great detail using combinatorial peptide libraries (4). The libraries contained nonapeptides which had been randomized at all positions except at a single residue. For each defined position, there were 19 variations (cysteine was not used), resulting in a total of 171 libraries (4). Only small (2- to 3-fold) differences in affinity (estimated from competitive binding of the libraries with bradykinin) and transport rates were observed between different defined amino acids at any

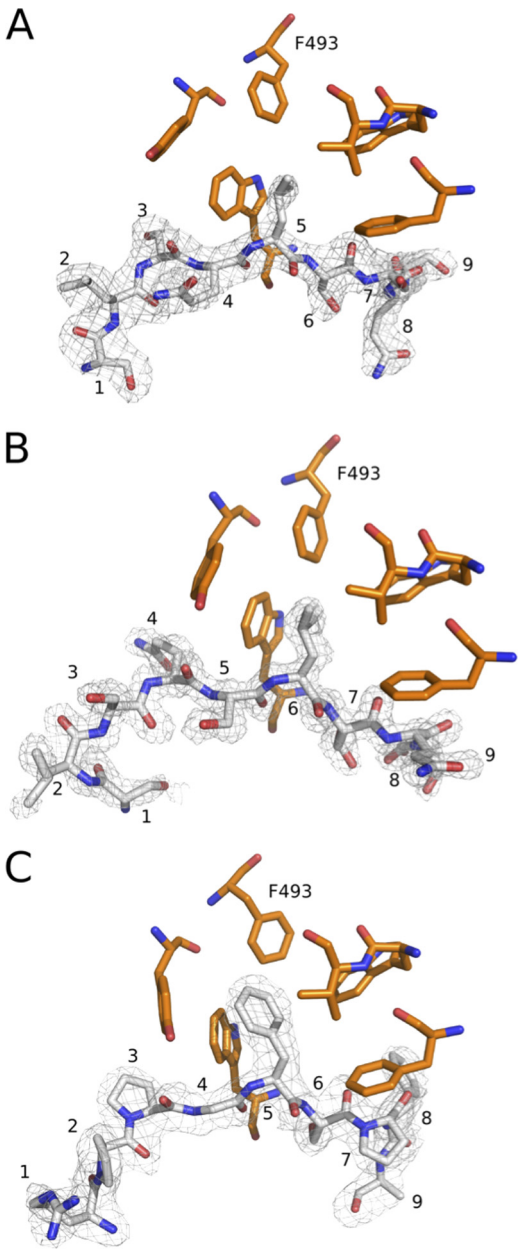

FIG. 1. Peptide binding to OppA* in different registers: SLSQL SSQS (A), SLSQSLSQS (B), and bradykinin (RPPGFSPFR) (C). The peptide ligands are in gray, with electron density ($2F_o$-F_c map) displayed as a gray mesh contoured at 1 σ. OppA* residues forming the single well-defined hydrophobic side chain pocket are in orange.

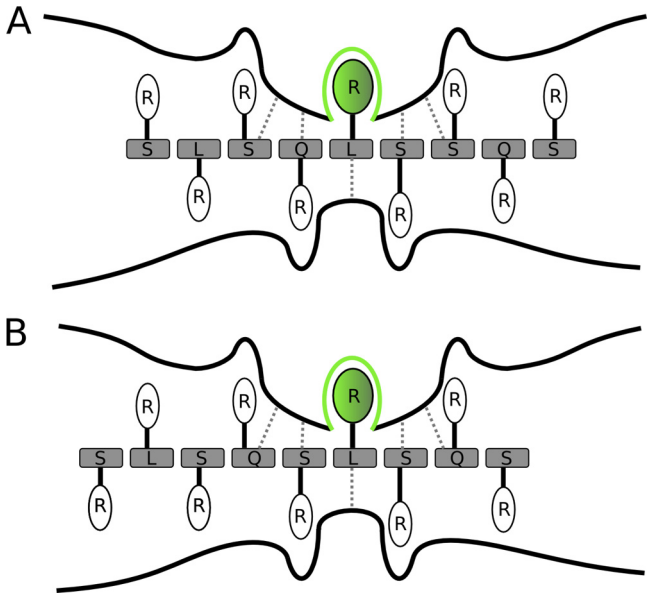

FIG. 2. Schematic drawing of the binding cavity of OppA with bound SLSQLSSQS (A) and bound SLSQSLSQS (B). The well-defined, hydrophobic side chain pocket is depicted in green and determines the position of the bound peptide. The central part of the cavity, forming hydrogen bonds to 5 residues of the bound peptide, is restricted in terms of volume, but the cavity opens up to a large space on either side. Unlike with OppA from *S.* Typhimurium, neither the N nor the C terminus of the peptide is fixed within OppA from *L. lactis*; the peptides can extend within the voluminous cavities on both sides.

position in the nonapeptide (4, 5). It was concluded that ligand binding to OppA* was indifferent to the peptide sequences. At that time, the available structure of OppA from *Salmonella* Typhimurium (16), a protein that accepts di- to pentapeptides, was used to explain the binding of nonameric and longer peptides to the lactococcal OppA (4, 11). It was assumed that nonapeptides would bind in the same register, with the N terminus being fixed and buried and the C terminus having 3 to 4 residues protruding from the binding pocket. We now unambiguously show that nonapeptides can bind in different registers within the 4,900-Å^3 binding cavity. The (most) hydrophobic amino acid in the central region of the peptide docks into the hydrophobic pocket of OppA and thus anchors the peptide in a specific register. The peptides of the combinatorial libraries had only one defined side chain, at position X, with all other positions being randomized. Peptides could have shifted the register by one or perhaps by multiple positions to avoid having an unfavorable, defined side chain in the hydrophobic pocket. In that way, differences in affinities are smoothened out when combinatorial libraries are used. A corollary of our model is that peptides with exclusively hydrophilic residues in the stretch of 5 amino acids that interact with the protein will be low-affinity ligands; changing one of these into a hydrophobic amino acid would have an enormous impact on the affinity. This is in agreement with earlier *in vivo* growth studies which showed that hydrophobic peptides were used before hydrophilic ones (7). Our work establishes that Opp from *L. lactis* uses a peptide-binding mechanism that has evolved the capacity to transport extremely long peptides, a capacity that is different from that of equivalent transporters in enteric bacteria. This mode of oligopeptide selection may hold in general for Gram-positive organisms that utilize milk proteins as their main source of nitrogen.

Protein Data Bank accession numbers. The final coordinates of the lactococcal OppA* protein, complexed with either SLSQLSSQS or SLSQSLSQS, have been deposited in the Protein Data Bank under accession numbers 3RYA and 3RYB, respectively.

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